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14. ABSTRACT
Metastatic spread of prostate cancer is the second leading cause of deaths of men in the United States. Although there are many ways to treat non-metastatic form of prostate cancer, only androgen-deprivation therapy is available for the extensive stage. Again, the cancer will often progress to an androgen refractory (independent), metastatic stage. Recent reports have suggested that the expression of VEGF-C and its receptor VEGFR-3 are directly correlated with lymph node dissemination in prostate cancer. This finding leads us to think that understanding the role of angiogenic molecules like VEGF-C, -D in molecular detail for lymphatic formation in prostate cancer will provide us the information regarding their relationship with lymph node metastasis. We have observed up regulation of VEGF-C in prostate cancer cells upon androgen withdrawal. Down regulation of IGF-IR pathway and SIRT-1 mediated activation of fork head transcription factor, FOXO-1 was the key regulatory mechanism identified for the androgen dependent regulation of VEGF-C. We have also established the orthotopic mouse model of prostate cancer for our future detail studies proposed in this grant.

15. SUBJECT TERMS
vascular endothelial growth factor-C, lymphangiogenesis, metastasis

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Introduction: Metastatic spread of prostate cancer is the second leading cause of deaths of men in the United States. Every year almost 40.000 American men die from prostate cancer of which more than 70% die due to complications of late stage tumors that metastasize to distant location. Lymph node metastasis is the early prognosis for the spread of this disease. Although there are many ways to treat non-metastatic form of prostate cancer like anti-androgen therapy, radical prostatectomy, radiotherapy and cytotherapy, only androgen-deprivation therapy is available for the extensive stage. Again, the cancer will often progress to an androgen refractory (independent), metastatic stage (*1-3*). However, the detailed molecular mechanism underlying the metastatic spread of this disease is poorly understood. Thus it has been difficult to develop effective treatments in this stage of prostate cancer. Recent reports have suggested that the expression of VEGF-C and its receptor VEGFR-3 are directly correlated with lymph node dissemination in prostate cancer (*4, 5*). This finding leads us to think that understanding the role of angiogenic molecules like VEGF-C, -D in molecular detail for lymphatic formation in prostate cancer will provide us the information regarding their relationship with lymph node metastasis. Thus, in this present study, the main focus will be to unravel the detail molecular mechanisms of these molecules that lead to the metastatic spread of prostate cancer to lymph node.

Body: Task1 described in the Statement of Work of our proposed application was as follows:

a. Develop a prostate cancer orthrotopic mouse model.

We successfully accomplished the goal by generating the orthotopic prostate cancer mouse models. Human prostate cancer models were generated by us using 8-week-old male athymic BALB/c nude mice (NCI). Exponentially growing human prostate cancer cell lines, LNCaP and PC-3 (ATCC), were used for implantation. Mice were anesthetized by isoflurane and a transverse incision was made in the lower abdomen. The bladder and seminal vesicles were then delivered through the incision to expose the dorsal prostate. LNCaP cells (~$2X10^6$ cells/50 µl medium) or PC-3 cells (~$3X10^5$ cells/50 µl medium) were injected under the prostatic capsule with a 26.5-gauge needle. The incision was closed with a running suture of 5-0 silk. For LNCaP cells, tumor formation (Figure 1) and lymph node metastases (Figure 2) were observed 100 days post-tumor injection. The incidence of tumor formation in this model was 70% and almost 50% of the tumor-bearing animals show paraaortic lymph node metastases. For the PC-3 model, we observed 100% incidence of tumor formation and paraaortic lymph node metastasis 45 days post-PC-3 implantation.

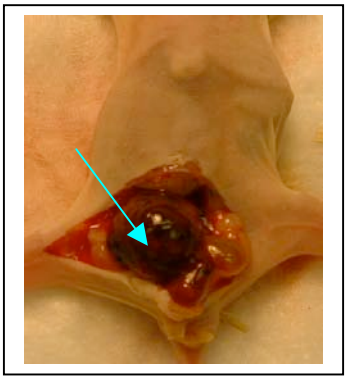


Figure 1: *LNCaP cells were injected into the prostate of athymic mice. Tumor was observed 100 days after implantation.*

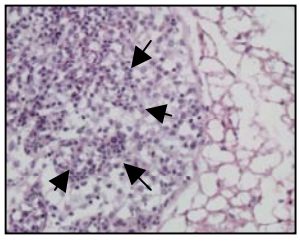


Figure 2: *H&E staining of the regional lymph node showing tumor cell (LNCaP) metastasis.*

Orthotopic xenograft tumor of human PC-3 cells expresses high level of VEGF-C as detected by *in situ* hybridization: Orthotopically developed PC-3 tumors and its surrounding stroma were harvested, paraformaldehyde-OCT fixed, and serially sectioned for *in situ* hybridization with a specific radio-labeled probe against VEGF-C. A significant upregulation of VEGF-C mRNA level was observed in the tumor compare to the normal prostate region adjacent to the tumor.

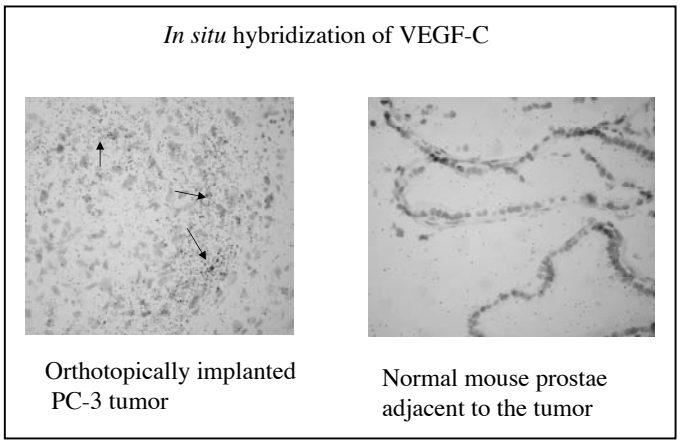


Figure 3: In situ hybridization with probe specific for VEGF-C in the orthotopically implanted PC-3 tumor and the normal prostate tissue adjacent to the tumor

b. Detection of lymphangiogenesis in prostate cancer and its relation to lymph metastasis.

We used antibody to detect podoplanin, a marker for lymphatic vessel, for our immunohistochemistry study to detect lymphatic vessel in human prostate tumor. Intratumoral lymphatic vessels were detected by us suggesting the evidence of lymphangiogenesis in human prostate tumor.

Currently, the study for understanding the relation of lymphangiogenesis to lymph node metastasis in prostate cancer is underway.

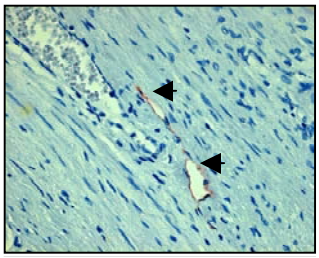


Figure 4: Podoplanin staining of human prostate cancer tissue (arrow head)

c. Determine the regulatory role of VEGF-C, VEGF-D in lymph angiogenesis

Our goal in this aim is to develop prostate cancer cell lines that express inducibly the siRNA of VEGF-C and VEGF-D. These cell lines will be used to detect lymphangiogenesis and lymph node metastasis in an orthotopic mouse prostate tumor model in the presence or absence of VEGF-C and VEGF-D.

The following results were obtained that have laid the foundation for the successful generation of stable LNCaP cell lines expressing the siRNA of VEGF-C in an inducible manner.

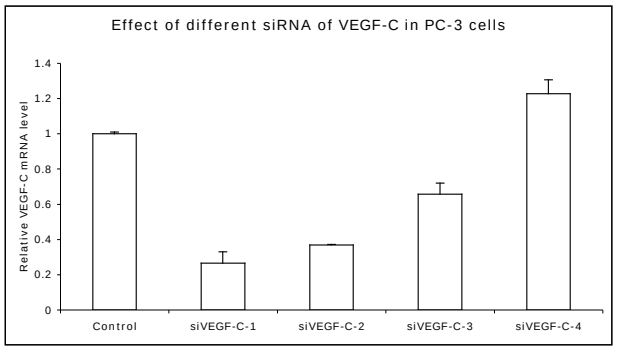


Figure 5: PC-3 cells were transiently transfected with four different siRNA for VEGF-C. Total RNA were isolated and subjected to real-time PCR to determine the relative VEGF-C mRNA level for each siRNA transfected PC-3 cells.

We initially tested four double stranded RNA sequences (from Dharmacon) for inhibition of VEGF-C expression. Of the four sequences, three sequences inhibited VEGF-C mRNA level

in PC-3 when transiently transfected for 48 hours (Figure 5). The double stranded RNA sequence that most effectively inhibited VEGF-C mRNA level are as follows. Sense sequence: CAACCGAGAAUUUGAUGAAUU and Antisense sequence: PUUCAUCAAAUUCUCGGUUGUU. This particular sequence has also been cloned into a pSUPER retro-virus vector and its efficacy tested in PC-3 cells (Figure 6). Our preliminary data suggest a two-fold reduction in VEGF-C mRNA in PC-3 cells after transfection with this pSUPER construct. At present we are trying to standardize the optimal conditions for this virus mediated VEGF-C siRNA approach for both PC-3 and LNCaP cells.

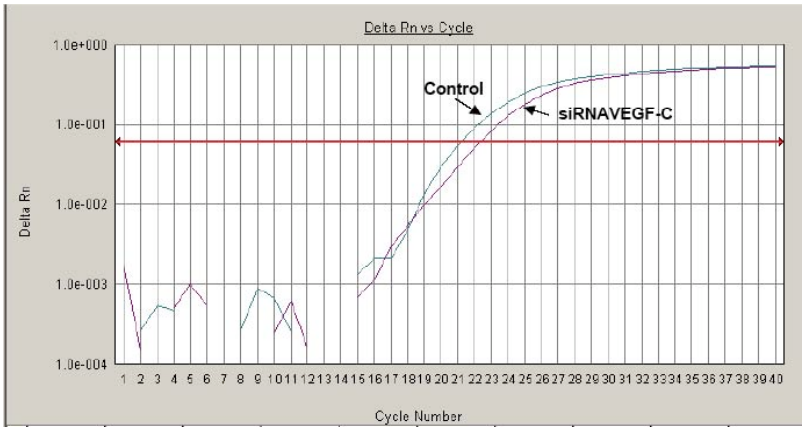


Figure 6: A representative picture of real-time PCR data showing almost two fold reduction in VEGF-C mRNA level of PC-3 cells when infected with retro virus containing pSUPER plasmid containing VEGF-C siRNA compare to control PC-3 cells.

We have also successfully cloned the VEGF-C siRNA sequence into the pSUPERIOR vector. This vector will produce siRNA of VEGF-C in an inducible way.

At present, we are working on to fulfill the following goals.

1. Preparation of stable LNCaP cell line that expresses Tet repressor:
2. Infection of pSUPERIOR into LNCaP-Tet cells
3. *In vivo* experiments for examining the effect of inhibition of the expression of VEGF-C and –D in prostate cancer.

Task 2. Elucidation of the molecular pathway that regulates the expression of VEGF-C in prostate cancer.

Transcriptional regulation of VEGF-C in prostate cancer

We have generated a significant amount of informations regarding the transcriptional control of VEGF-C in prostate cancer. They are as follows:

Previous reports suggest a positive correlation between VEGF-C expression and the metastatic potential of human prostate cancer cells (*5*) (*4*). In order to test whether a greater metastatic potential correlates with higher expression levels of VEGF-C, VEGF-C protein levels were compared by ELISA between the more metastatic PC-3 and less metastatic LNCaP cell lines (Figure 7A) as well as among different sublines of LNCaP cells with varying metastatic potentials (Figure 7B). A significant increase in VEGF-C protein level was observed in PC-3 compared to LNCaP. Interestingly, among different LNCaP sublines, the most metastatic LNCaP-LN3 cell line expresses more VEGF-C as compared to the parental LNCaP and LNCaP-pro5 cell lines. LNCaP-pro5, intermediate in terms of metastatic potential, expressed a VEGF-C mRNA level greater than that of LNCaP, but lower when compared to that of LNCaP-LN3.

It is known that androgen deprivation is associated with the gradual transition of prostate cancer from the androgen-dependent stage to the androgen refractory stage. Furthermore, the tumor recurs with a more metastatic phenotype (*6*). This data prompted us to investigate whether androgen could regulate VEGF-C expression in LNCaP cells. LNCaP cells were grown in media with charcoal-dextran-treated serum for 48 hr, 72 hr, 96 hr, and 120 hr. Using real-time PCR, we observed a time dependent up regulation of VEGF-C mRNA across the time points tested (Figure 7C), suggesting a negative regulation of VEGF-C expression by androgen.

Recently, Arnold *et.al.* described a functional correlation between androgen and IGF-IR expression in prostate cancer cells (*7*). They showed that androgen stimulation of LNCaP cells cultured in media containing charcoal dextran-treated serum led to an increase in IGF-IR protein expression. In another report, Plymate *et.al*. showed that the progression from an organ-confined tumor to metastatic prostate cancer was characterized by a deregulation of the androgen receptor and a decrease in IGF-IR protein expression (*8*). Since androgen withdrawal also increases VEGF-C mRNA levels, we investigated whether IGF-IR also influenced regulation of VEGF-C transcription. Our real-time PCR data (Figure 7D) suggest an 8-10 fold increase in VEGF-C mRNA levels when blocking antibody specific for the IGF-IR receptor inhibited IGF-IR signaling. The increase in VEGF-C mRNA by blocking antibody specific for IGF-IR has also been observed in another androgen dependent prostate cancer cell line 22RV1 (data not shown) suggesting this increase in VEGF-C mRNA after androgen withdrawal is not a particular phenomenon of LNCaP cells.

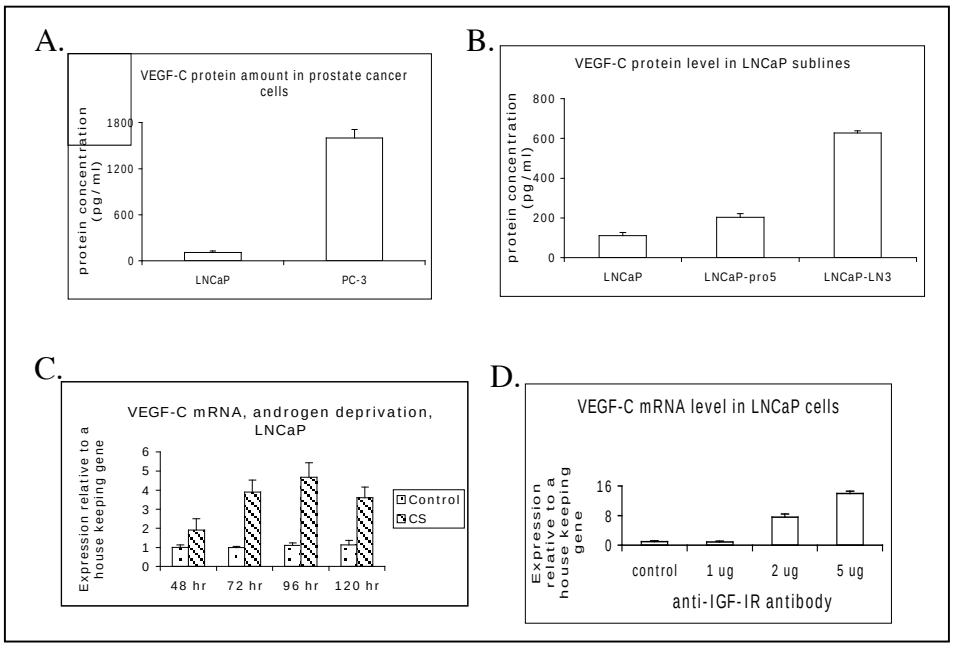


FIGURE *7*

A and B*. Protein amount of VEGF-C in prostate cancer cell lines by ELISA: The amount of VEGF-C in the conditioned media of (A) LNCaP and PC-3 cells and (B) LNCaP, LNCaP-Pro5 and LNCaP-LN3 cells was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using specific antibody targeting VEGF-C. The data presented are the mean of two independent experiments.*

C. *Lncap cells were cultured in charcoal-dextran treated serum containing media for 48 hr, 72hr, 96 hr and 120 hr. total RNAs were obtained in each time point followed by RQ-PCR using primers specific for vegf-c and 36b4 (internal control). the data showed here is the average of three independent results.*

D. *LNCaP cells were grown in serum starved condition media for 24 hr, treated with anti-IGF-IR blocking antibody at different concentrations (1mg/ml, 2mg/ml and 5mg/ml respectively) for overnight. Normal mouse IgG (5mg/ml) was used as control. Real time PCR was performed using primers of VEGF-C and 36B4 (internal control) with total RNA isolated from cells. The data here represents the* mean of three different experiments.

FOXO-1 is the potential transcription factor for VEGF-C and is activated by SIRT-1: Forkhead transcription factors become functionally active due to the down-regulation of IGF-IR signaling. PKB or AKT activation by the IGF-IR-phosphoinositide-3 kinase (PI3K) pathway leads to the phosphorylation of forkhead transcription factors, causing them to be retained in the cytoplasm (*9-13*). Therefore, inhibition of the IGF-IR signaling pathway should lead to decreased phosphorylation of forkhead transcription factors, allowing them to enter the nucleus and promote the transcription of their target gene. Using the Genomatix software available on the internet (http://www.genomatix.de), we searched the known promoter region of VEGF-C for potential binding sites of transcriptional regulators and found several for the forkhead transcription factor, FOXO-1. To study whether FOXO-1 could indeed up-regulate VEGF-C transcription, we transiently over-expressed FOXO-1 in LNCaP cells and then used real-time PCR to monitor VEGF-C mRNA levels (Figure 8A). With the transient over-expression FOXO-1, we found a significant increase in VEGF-C mRNA levels.

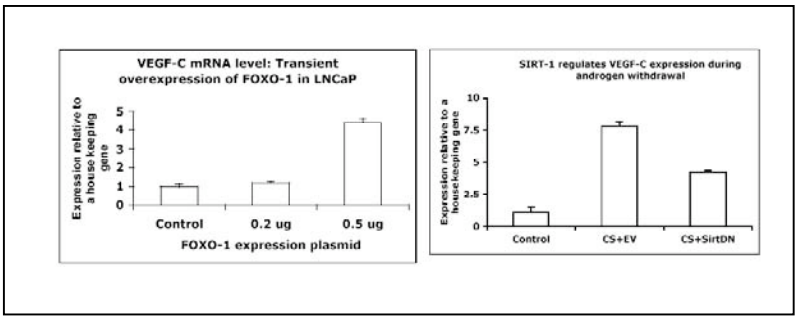


Figure 8. *FOXO-1 with SIRT1 upregulates VEGF-C transcription:*
A. *LNCaP cells transiently transfected with pcDNA3-Flag-FOXO-1 plasmid at 0.2 µg and 0.5 µg for 48 hr, total RNA were subjected to real time PCR using specific primers of VEGF-C and 36B4 (internal control).*
B. *Over expression of dominant negative form of SIRT1 decrease the VEGF-C mRNA level: LNCaP cells transiently transfected with plasmid expressing dominant negative form of SIRT1 in the presence or absence of anti-IGF-IR antibody (5µg/ml). Total RNA was extracted from LNCaP cells and mRNA level of VEGF-C was determined by RQ-PCR using primers specific for VEGF-C and 36B4 (internal control).*

Various biological functions controlled by FOXO-1 include cell cycle arrest, detoxification of reactive oxygen species, repair of damaged DNA, and apoptosis (*13*). Recent reports suggest that the presence of SIRT1 (mammalian homolog of yeast silencing information regulator 2 [Sir2] gene) enhances the expression of the FOXO target genes involved in stress resistance but diminishes the expression of pro-apoptotic genes like Fas ligand and BIM. Sir2 has been shown to play a role in increasing the life span of yeast and *Caenorhabditis elegans* and is conserved throughout evolution. It is a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase. Forkhead transcription factors have been shown to be targets of SIRT1. Deacetylation of FOXO transcription factors by SIRT1 is specifically required for the transcription of stress resistance genes, such as genes that control the repair of damaged DNA (GADD45) (*14-17*). The VEGF family is known to be involved in lymphangiogenesis, angiogenesis, and survival. We therefore studied the involvement of SIRT1 in the FOXO-1 dependent transcription of VEGF-C. We found abrogation FOXO-1 dependent VEGF-C transcription in LNCaP cells when the dominant negative form of SIRT1 was transiently overexpressed (Figure 8B). This result is significant for two reasons. First, it provides a mechanistic explanation for how FOXO-1, activated by SIRT1, can act to transcribe VEGF-C. Finally, it also indicates that VEGF-C is another target gene for SIRT1.

Androgen deprivation causes nuclear translocation of FOXO-1: Androgen dependent prostate cancer cell lines, LNCaP and 22RV1 were cultured either in the presence of 10% FBS containing media or charcoal stripped serum (androgen ablated) containing media for 96 hr. FOXO-1 protein localization was determined for both of these conditions by immunocytochemistry using an antibody specific for FOXO-1. Cells were

fixed for 20 min with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Cells were washed three times with 0.05% Triton-X-100 in PBS and permeabilized with 95% ethanol for 5 minutes. The cells were then be blocked for 1hr with 1% BSA-PBS prior to incubation with the FOXO-1 antibody diluted in PBS for 1 hr at 37^{0}C. After the primary antibody staining, cells were washed again three times with 0.05% Triton-X-100 in PBS followed by incubation with the FITC-tagged secondary antibody for 1 hr at 37^{0}C. After three final washes with 0.05% Triton-X-100 in PBS, the cells were visualized with a fluorescence microscope using DAPI for nuclear staining. As shown in figure 9, a strong cytoplasmic staining for FOXO-1 was observed in both LNCaP and 22RV1 cells when cultured in 10% FBS containing media. A significant increase in the nuclear staining of FOXO-1 was detected in both the cell lines when cultured in androgen-depleted media (figure 9).

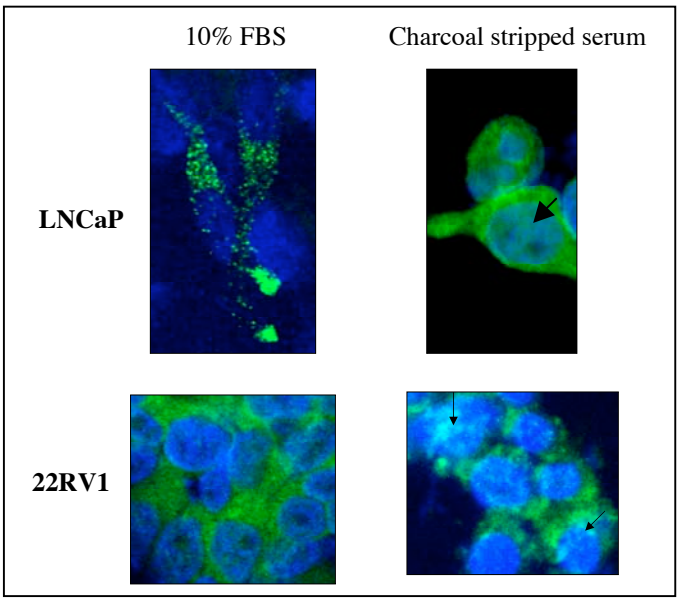


Figure 9: Immunocytochemistry result with anti-FOXO-1 antibody. Upper panel represents the LNCaP cells cultured in the presence of 10% FBS or charcoal stripped serum. Lower panel represents the 22RV1 cells grown in the same two different serum conditions.

NKX3.1 downregulates VEGF-C mRNA level in LNCaP cells: Loss of prostate specific homeobox protein NKX3.1 is often correlated with the progression of prostate cancer (*18, 19*). Using the same Genomatix software mentioned above, we also identified five potential NKX3.1 binding sites in the promoter region of VEGF-C. We were able to significantly decrease NKX3.1 synthesis in LNCaP cells using siRNA specific for NKX3.1. A significant up-regulation of VEGF-C mRNA levels was observed by real-time PCR in LNCaP cells previously transfected with siRNA of NKX3.1 (Figure 10), suggesting the importance of NKX3.1 for VEGF-C transcription.

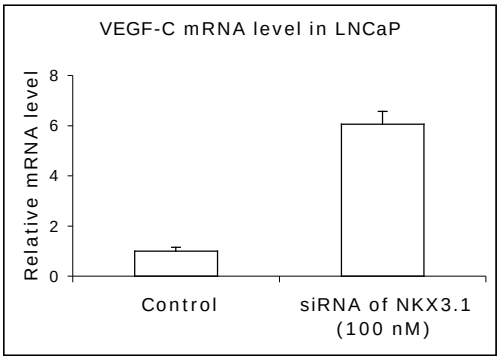
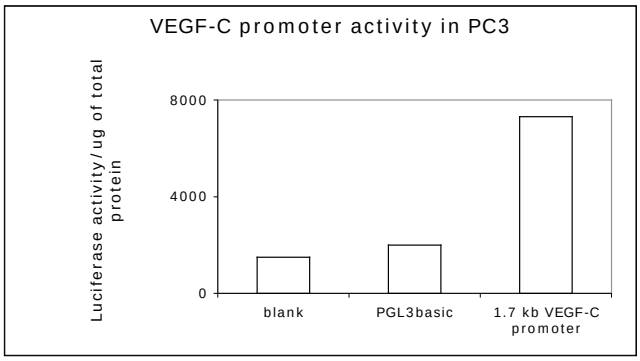


Figure 10: *LNCaP cells transiently transfected with siRNA of NKX3.1 (100 nM) for 48 hr, total RNA were subjected to real time PCR using specific primers of VEGF-C and 36B4 (internal control).*

Development of VEGF-C promoter-luciferase construct: A 1.7 kb fragment of VEGF-C promoter region from the transcription start site was amplified by PCR using specific primers from the genomic DNA isolated from the PC-3 cell line. This region of VEGF-C promoter contains the four out of five NKX3.1 binding sites. The amplified PCR fragment was then cloned into the pGL3-basic vector. The plasmid containing 1.7 kb VEGF-C promoter was then transfected into PC-3 cell line and luciferase activity was measured from the cell lysate using the luciferase assay kit (Promega). Luciferase activity in cells transfected with the 1.7 kb VEGF-C promoter was compared to that of PC-3 cell lysates transfected with vector only. The luciferase activity results were also normalized with the total protein reading of all cell lysates. A significant increase in luciferase activity was observed for the 1.7 kb VEGF-C promoter

containing construct, as compared to the control vector (Figure 11). This construct will be an important reagent for the study of NKX3.1 regulation of VEGF-C transcription.

Figure 11: VEGF-C Promoter Activity in PC-3 cell line: Plasmid DNA containing the 1.7 kb VEGF-C promoter fused with luciferase reporter gene was transiently transfected in PC-3 cell line. Total luciferase activity in the cells was measured by a chemiluminescent reader.



Key Research Accomplishments:

1. We have successfully developed orthotopic mouse models of human prostate cancer that will enable us to study the role of VEGF-C in prostate cancer metastasis.
2. We have delineated a molecular pathway that is responsible for the VEGF-C transcriptional upregulation in prostate cancer in the androgen-ablated condition.
3. We have successfully cloned different promoter regions of VEGF-C in a reporter-luciferase vector that will help us to study molecular regulation of VEGF-C.
4. We have made significant progress in order to generate the prostate cancer cell lines that will express siRNA of VEGF-C in an inducible manner. This cell lines will help to understand the function of VEGF-C in prostate cancer metastasis.

Reportable Outcome:

Published paper: Li J, Wang E, Rinaldo F, **Datta K**. (2005) Up-regulation of VEGF-C by androgen depletion: the involvement of IGF-IR-FOXO pathway. *Oncogene* 24(35), 5510-20.

Abstract presented: Li J, Wang E, Rinaldo F, **Datta K**. (2006) Regulation and Function of Vascular Endothelial Growth Factor-C in Prostate Cancer. *Prostate Spore Meeting*, Houston, Texas.

Plasmids generated: VEGF-C promoter luciferase expression construct in pGL3basic. siRNA of VEGF-C in pRetroSuper and pRetroSupirior.

Developed the orthotopic mouse model of human prostate cancer in immunocompromised mice.

Conclusion: One major concern of the reviewers in our proposal was the possibility of the development of orthotopic mouse model of human prostate cancer. This model was crucial in order to understand the involvement of lymphangiogenesis in prostate cancer metastasis. With the help of Dr. Donald J. Tindall, a well-known researcher in prostate cancer research, we have developed the orthotopic prostate cancer mouse model by implanting LNCaP and PC3 cells into the dorso-lateral lobe of immunocompromised mouse prostate. We have also delineated a possible molecular mechanism of VEGF-C upregulation in prostate cancer cell upon androgen withdrawal.

Since various published results and also our preliminary results suggest that Vascular endothelial growth factor-C (VEGF-C) is correlated with prostate cancer metastasis (*4, 5*), understanding the molecular mechanism of VEGF-C synthesis and its role in prostate cancer metastasis will be important for developing novel therapies for the metastatic stage of prostate cancer. In this respect, we are encouraged by the results that we observed in the first one-year period of our proposal.

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Upregulation of VEGF-C by androgen depletion: the involvement of IGF-IR-FOXO pathway

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Androgen ablation therapy is eventually followed by a more metastatic and androgen-refractory stage of prostate cancer. The detailed molecular mechanism of this gradual transition is not clearly understood. Recent reports correlate the high abundance of vascular endothelial growth factor-C (VEGF-C) to the lymph node metastasis seen in human prostate cancer (Tsurusaki *et al*., 1999). In this study, we report that androgen ablation in LNCaP cells augment the transcriptional upregulation of VEGF-C and the downregulation of the IGF-IR pathway, due to androgen withdrawal, is a potential mechanism for this observed VEGF-C transcription. Forkhead transcription factor FOXO-1, activated by SIRT-1, was identified as the downstream molecule within this pathway. Furthermore, the VEGF-C-induced increase of Bag-IL expression in LNCaP cells suggests that VEGF-C plays a role in the androgen-independent reactivation of the androgen receptor, resulting in androgen-refractory prostate cancer growth.
Oncogene (2005) **24,** 5510–5520. doi:10.1038/sj.onc.1208693; published online 9 May 2005

Keywords: VEGF-C; prostate cancer; metastasis; androgen; IGF-IR

Introduction

Prostate cancer is the second leading cause of cancer-related mortality for men in the United States (Greenlee *et al*., 2000). The established protocol for treating the advanced stage of this disease is surgical castration and/or chemotherapy to eliminate circulating androgens (Epstein *et al*., 1996). Activation of the androgen receptor by androgens is required for cell proliferation in the prostate. Therefore, androgen ablation therapy is initially effective in inhibiting cancer cell growth in most patients. However after a period of time, the tumor recurs in an androgen-refractory manner with a more aggressive and metastatic phenotype, ultimately leading to patient death (Kyprianou *et al*., 1990; Denis and Murphy, 1993; Oh and Kantoff, 1998; Isaacs, 1999). As the detailed molecular mechanism underlying the metastatic spread of prostate cancer is poorly understood, it has therefore been difficult to develop effective treatments at this stage of the disease. Initially, the concept of clonal selection was thought to explain the progression of the disease but subsequent molecular investigations of the androgen receptor (AR) provided a more mechanistic approach to understanding the metastatic progression of prostate cancer during androgen deprivation. Possible mechanisms for AR re-activation include AR gene mutation, gene amplification, the involvement of coregulators, and crosstalk between different signal transduction pathways (Sadar *et al*., 1999).

Apart from the possibilities discussed above, the involvement of other pathological mechanisms cannot be dismissed. In this regard, angiogenesis represents an effective target mechanism (Stewart *et al*., 2001; Nicholson and Theodorescu, 2004; Gustavsson *et al*., 2005). Angiogenesis is a process by which new vasculature is developed from pre-existing vessels and it is particularly important for the growth and metastasis of most human tumors (Folkman, 1971; Holash *et al*., 1999; Carmeliet, 2000). There have been previous reports that androgens may potentially modulate angiogenesis in animal and human prostate tumors by increasing vascular endothelial growth factor (VEGF) gene transcription (Stewart *et al*., 2001). As a result, an initial and rapid vascular regression in the tumor occurs after hormone withdrawal. Interestingly, progression of prostate cancer to an androgen-independent stage is also associated with increased angiogenesis. The lack of angiogenic regulation by androgens may be an additional factor accompanying the transition to this stage of androgen resistance (Jain *et al*., 1998). Therefore, it is important to identify any regulatory dysfunction of angiogenic growth factors during androgen-refractory stage of prostate cancer.

VEGF-A is the founding member of closely related cytokines that exert critical functions in vasculogenesis, pathologic and physiologic angiogenesis and lymphangiogenesis (Dvorak *et al*., 1995; Dvorak, 2000; Ferrara, 2001). Other members of the VEGF family include VEGF-B, VEGF-C, VEGF-D, VEGF-E (orf virus VEGF) and placental growth factor (P1GF) (Chilov

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et al., 1997; Enholm *et al*., 1997; Achen *et al*., 1998, 2002; Olofsson *et al*., 1999; Dvorak, 2000; Carmeliet *et al*., 2001). All of these ligands mainly bind to and activate three receptor tyrosine kinases (TRK) to initiate their downstream signaling. These TRKs include: VEGFR-l/Flt-1, VEGFR-2/Flk-l/KDR and VEGFR-3/Flt4. VEGF-A binds to VEGFR-1 and VEGFR-2 whereas VEGF-C and -D bind to VEGFR-2 and VEGFR-3. VEGF-B binds only VEGFR-1 and VEGF-E binds only VEGFR-2 (de Vries *et al*., 1992; Dvorak *et al*., 1995; Hatva *et al*., 1995; Kaipainen *et al*., 1995; Joukov *et al*., 1996; Dvorak, 2000; Kubo *et al*., 2000; Veikkola *et al*., 2000). There is also a fourth nontyrosine kinase receptor called neuropilin-1 that mainly binds VEGF-A (Soker *et al*, 1998).

Among the angiogenic cytokines, VEGF-C draws special attention because of its potential involvement in the lymph node metastasis of several cancers including – colorectal cancer, human pancreatic endocrine tumors, esophageal carcinomas, head and neck squamous cell carcinoma, uterine cervical cancer, primary non-small-cell lung cancer, gastric carcinoma, and laryngeal squamous carcinoma (Yonemura *et al*., 1999; Hashimoto *et al*., 2001; Kimura *et al*., 2003; Neuchrist *et al*., 2003; Jia *et al*., 2004; Sipos *et al*., 2004; Wang *et al*., 2004). Recently, Tsurusaki *et al*. reported a significant correlation between the expression of VEGF-C and lymph node dissemination in human prostate carcinoma (Tsurusaki *et al*., 1999). They found an increased number of vessels positive for VEGFR-3, the VEGF-C receptor, in surrounding stromal tissue of VEGF-C positive prostatic carcinoma cells (Tsurusaki *et al*., 1999; Zeng *et al*., 2004). VEGF-C has a high degree of sequence homology with VEGF-A. It also has eight-conserved cysteine residues involved in intra- and intermolecular disulfide bonding. The cysteine-rich COOH-terminal increases the length of VEGF-C by half as compared to other VEGF family members. VEGF-C, like VEGF-A, has alternative spliced forms. VEGF-C mRNA is first translated into a precursor that, after secretion, undergoes cell associated proteolytic processing. The mature form of VEGF-C is attained after the proteolytic removal of its NH_2- and COOH-terminal extensions. Mature form has increased binding affinity towards VEGFR-3 by approximately 400-fold. It can also bind to VEGFR-2 while the unprocessed form of VEGF-C can bind only VEGFR-3 (Lee *et al*., 1996; Pepper, 2001). The mature form can induce endothelial cell proliferation and migration as well as vascular permeability (Joukov *et al*., 1997). Despite these reports, the individual role of each receptor on VEGF-C stimulation is not yet clear. In contrast to VEGF-A, VEGF-C is not regulated by hypoxia (Enholm *et al*., 1997). The expression profile of VEGF-C and its binding to VEGFR-3 suggests its involvement in the development of the lymphatic system or lymphangiogenesis in the tumor microenvironment.

Cytokines such as VEGF-C are multifunctional in nature. Therefore, increased expression of VEGF-C in the tumor microenvironment will trigger an enhancement of many cellular functions and deregulate the balance between different physiological processes. One possible function of VEGF-C in the tumor microenvironment mentioned before is that of lymphangiogenesis, providing a potential route for tumor cell dissemination to the lymph node. Because, this may not be the only function of VEGF-C-promoting metastasis, it will be interesting to study the potential functions of VEGF-C, apart from lymphangiogenesis, that may be responsible for the progression of prostate cancer to the androgen-refractory condition. It has been reported that VEGF-C can stimulate proliferation and migration of Kaposi's sarcoma cells (Marchio *et al*., 1999) and also proliferation and survival for leukemia (Dias *et al*., 2002). Again, lack of lymphangiogenesis observed in uveal melanoma despite its high expression of VEGF-C, suggests a different function in cancer progression (Clarijs *et al*., 2001).

In the androgen-dependent prostate cancer cell line LNCaP, we have observed VEGF-C expression. Furthermore, androgen withdrawal upregulates VEGF-C expression in LNCaP cells. Our data suggest that the androgen receptor (AR)-IGF-IR axis is important for the observed VEGF-C transcriptional upregulation in LNCaP. Again, an increase in Bag-1L protein level by VEGF-C was observed in LNCaP cells that may provide an explanation for activation of androgen receptor by VEGF-C in low androgen concentration.

Results

VEGF-C is highly expressed in metastatic prostate cancer cell line

Previous reports suggest a positive correlation between VEGF-C expression and the metastatic potential of human prostate cancer cells. In order to test whether a greater metastatic potential correlates with higher expression levels of VEGF-C, we used real-time PCR to quantify VEGF-C mRNA levels in three prostate cancer cell lines: PC-3, DU145, and LNCaP (Figure 1a). The VEGF-C mRNA level in each cell line was normalized to the housekeeping gene 36B4. Interestingly, the VEGF-C mRNA level in PC-3, the cell line with the highest metastatic potential, was 250-fold higher than that of LNCaP, the cell line with the lowest metastatic potential. DU145, intermediate in terms of metastatic potential, expressed VEGF-C mRNA at levels 150-fold greater than that of LNCaP but lower when compared to that of PC-3. Although VEGF-A mRNA levels were only two fold higher in the PC-3 cell line and four fold higher in the DU145 with respect to LNCaP, the levels were clearly not as high as VEGF-C (Figure 1b). Protein levels of VEGF-C in PC-3 and LNCaP cell lines were also in line with the real-time PCR data when tested by ELISA (Figure 1c). Taken together, these results pointed to the potential importance of VEGF-C in prostate cancer metastasis.

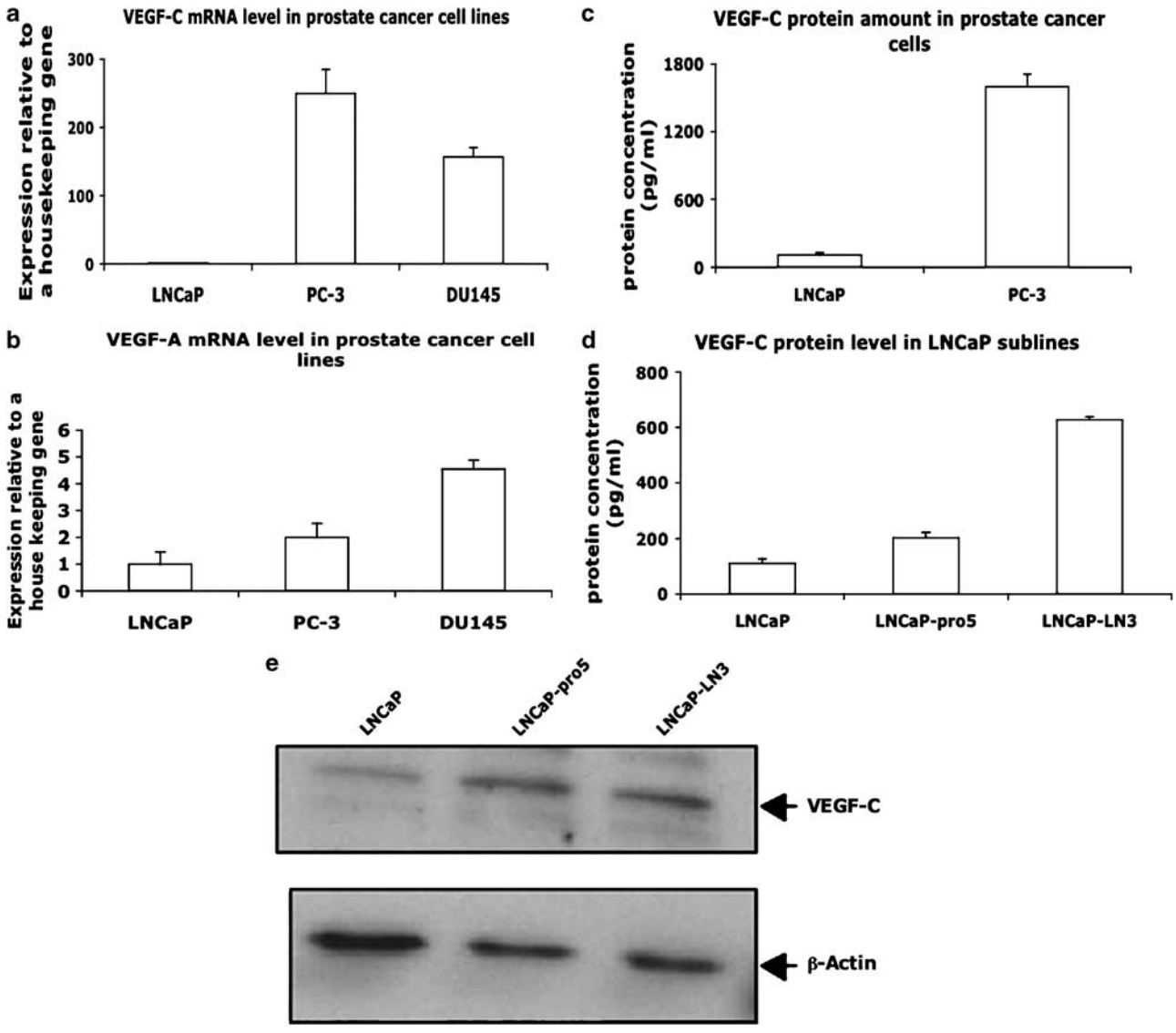

Figure 1 (**a** and **b**) mRNA level of VEGF-C and VEGF-A in LNCaP, DU145 and PC-3 cell lines. Real-time PCR was performed using specific primers for (**a**) VEGF-C, and (**b**) VEGF-A with total RNA isolated from three prostate cancer cell lines LNCaP, DU145 and PC-3. The data represented here are the average of three independent results. (**c**) Protein amount of VEGF-C in prostate cancer cell lines by ELISA. The amount of VEGF-C in the conditioned media of LNCaP and PC-3 cells was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using specific antibody targeting VEGF-C. The data presented are the mean of two independent experiments. (**d** and **e**) VEGF-C protein level in LNCap sublines. (**d**) The amount of VEGF-C in the conditioned media of LNCaP, LNCaP-Pro5 and LNCaP-LN3 cells was measured by ELISA using specific antibody against VEGF-C. The data presented are the mean of two independent experiments. (**e**) Whole cell extract was collected from LNCaP, LNCaP-Pro5 and LNCaP-LN3 cells. Western blot against VEGF-C (upper panel) antibody and β-actin (lower panel) was performed

The more metastatic subline LNCaP expresses higher levels of VEGF-C

VEGF-C protein levels were also tested in LNCaP-Pro5 and LNCaP-LN3, two different sublines of LNCaP with a greater metastatic potential than the parental cell line (Pettaway *et al*., 1996). We performed ELISA to quantitate the VEGF-C protein amount in the conditioned media of different LNCaP sublines (Figure 1d). The most metastatic subline LNCaP-LN3 expressed highest amount of VEGF-C. LNCaP-pro5 was intermediate in terms of metastatic potential among the sublines and expressed more VEGF-C protein than the parental cell line but less compared to LNCaP-LN3. Western blot results (Figure 1e) also show higher VEGF-C protein levels in both LNCaP-Pro5 and LNCaP-LN3 compared to the parental LNCaP line. These results again suggested the potential involvement of VEGF-C in metastasis.

Androgen negatively regulates VEGF-C levels in LNCaP cells

It is known that androgen deprivation is associated with the gradual transition of prostate cancer from the androgen-dependent stage to the androgen-refractory stage. Furthermore, the tumor recurs with a more

metastatic phenotype. This prompted us to investigate whether androgen can regulate VEGF-C expression in LNCaP cells. LNCaP cells were grown in media with charcoal–dextran-treated serum (reduced androgen-containing serum) for 48, 72, 96, and 120 h. Using real-time PCR, we observed a time-dependent upregulation of VEGF-C mRNA across the time points tested (Figure 2a), suggesting a negative regulation of VEGF-C expression by androgen. The value of VEGF-C mRNA level at each time point was normalized with the value of the house keeping gene 36B4. This result was confirmed when the upregulation of VEGF-C mRNA level was significantly inhibited after the addition of synthetic androgen (R1881) to the charcoal-treated media at the 48-h time point (Figure 2b). Additionally, the negative regulation of VEGF-C by androgen appears specific because a different profile was observed for VEGF-A mRNA. VEGF-A mRNA levels decreased in the absence of androgen but increased significantly when R1881 was added to the media (Figure 2c).

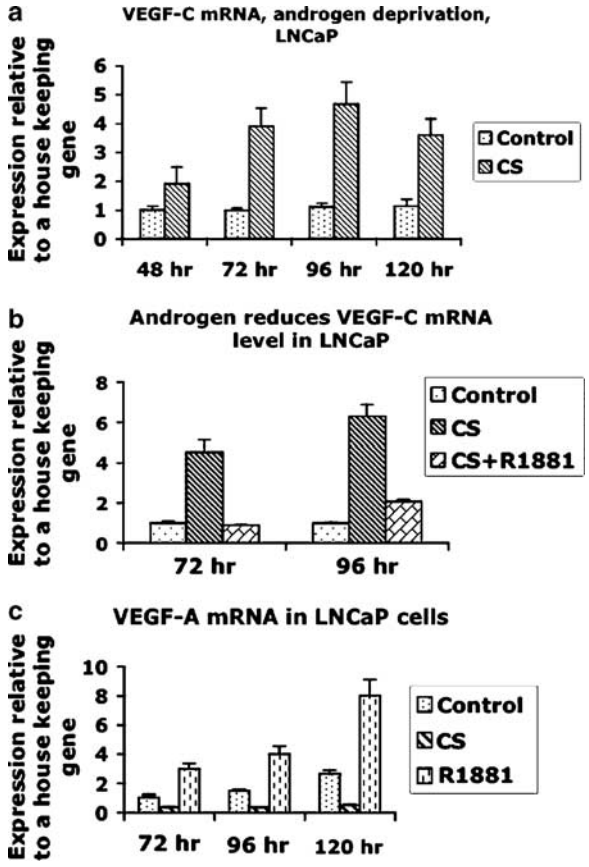

Figure 2 Androgen depletion increases VEGF-C mRNA level but decreases VEGF-A mRNA in LNCaP cells. (**a**) LNCaP cells were cultured in charcoal–dextran-treated serum-containing media (designated as CS in the figure) for 48, 72, 96 and 120 h. Total RNAs were obtained in each time point followed by RT–PCR using primers specific for VEGF-C and 36B4 (internal control). For the control experiment, LNCaP cells were cultured in the normal serum-containing media for the same time points described above and VEGF-C mRNA level was determined by RT–PCR. The data showed here are the average of three independent results. (**b** and **c**) LNCaP cells were first cultured in charcoal–dextran-treated serum-containing media (designated as CS in the figure) for 48 h. R1881 (1 μM) was then added to the media and the total RNA was isolated at the different time points with respect to the cells cultured in charcoal-stripped media. RT–PCR was performed with the total mRNA by using primers specific for VEGF-C (**b**) and specific for VEGF-A (**c**). 36B4 mRNA levels were monitored in every experiment for internal control. The data showed here are the average of three independent results

Functional inhibition of insulin-like growth factor-I (IGF-IR) signaling upregulates VEGF-C mRNA level

Recently, Arnold *et al*. (2005) described a functional correlation between androgen and IGF-IR expression in prostate cancer cells. They showed that androgen stimulation of LNCaP cells cultured in media containing charcoal–dextran-treated serum led to an increase in IGF-IR protein expression. We also observed the similar regulation of androgen for IGF-IR expression in LNCaP as shown in (Figure 3a). This result suggests the possible regulation of IGF-IR synthesis by androgens. In another report, Plymate *et al*. (2004) described that the progression from an organ-confined tumor to metastatic prostate cancer was characterized by a deregulation of the androgen receptor and a decrease in IGF-IR protein expression. These two findings suggest that decreasing IGF-IR levels plays a role on the progression of prostate cancer to its androgen-refractory, metastatic stage. Since androgen withdrawal also increases VEGF-C mRNA levels, we investigated whether IGF-IR also influenced regulation of VEGF-C transcription. Our real-time PCR data (Figure 3b) suggest a 8–10-fold increase in VEGF-C mRNA when blocking antibody specific for the IGF-IR receptor (Keller *et al*., 1993; De Meyts *et al*., 1995; Datta *et al*., 2000; Schlessinger, 2000) inhibited IGF-IR signaling. We also observed the increase in VEGF-C mRNA level in both the LNCaP-Pro5 and LNCaP-LN3 cells by inhibiting IGF-IR signaling (Figure 3c and d). The reason for not detecting the 8–10-fold increase in VEGF-C mRNA like parental LNCaP cell line was probably due to the fact that these two cell lines are more metastatic than LNCaP cells and already express higher level of VEGF-C (Figure 1d). Again, we did not observe any significant increase in VEGF-A mRNA in LNCaP cells when we inhibited the IGF-IR pathway suggesting a VEGF-C-specific phenomenon (Figure 3e). These results, therefore, correlate a possible link between androgen withdrawals, a decrease in IGF-IR protein expression, and VEGF-C upregulation in prostate cancer cells.

FOXO-1 is the potential transcription factor for VEGF-C and is activated by SIRT-1

Forkhead transcription factors become functionally active due to the downregulation of IGF-IR signaling. PKB or AKT activation by the IGF-IR-phosphoinositide-3 kinase (PI3K) pathway leads to the phosphorylation of forkhead transcription factors, which retains them in the cytoplasm (Borkhardt *et al*., 1997; Kops and

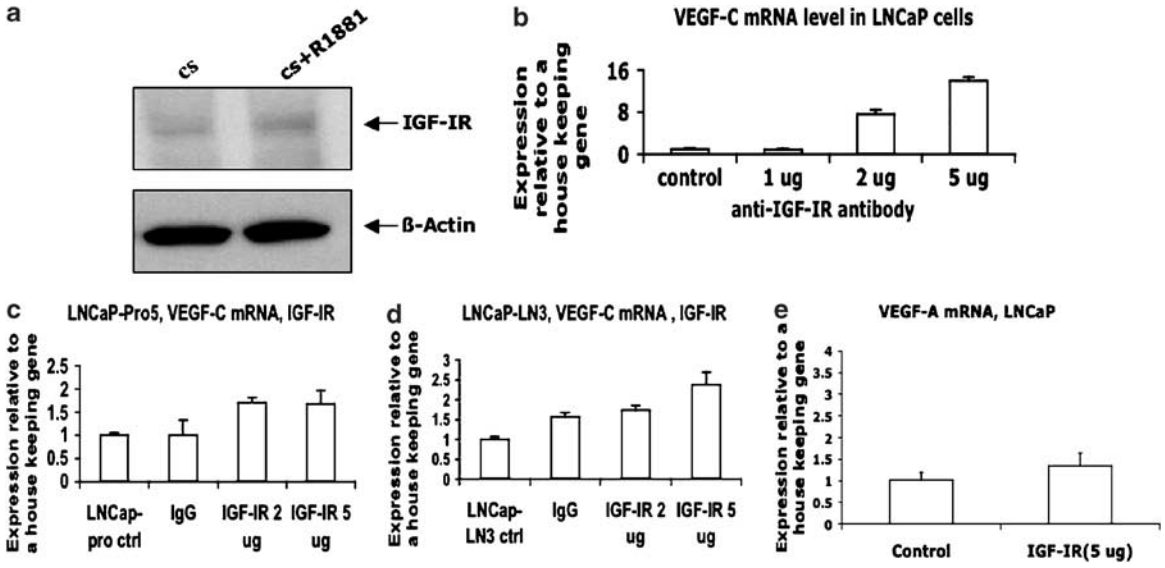

Figure 3 (**a**) Androgen increases the IGF-IR protein level in LNCaP. LNCaP cells were grown in the charcoal–dextran-treated serum-containing media (designated as CS in the figure) for 48 h, then R1881 (1 μM) was added for 24 h. Whole cell extracts were subjected to Western blot using anti-IGF-IR antibody. Protein level of IGF-IR was increased in presence of R1881 (upper panel), β-actin was used as internal control (lower panel). (**b**–**d**) Blocking IGF-IR signaling increases VEGF-C mRNA level in LNCaP, LNCaP-Pro5, and LNCaP-LN3 cells. LNCaP, LNCaP-Pro5, and LNCaP-LN3 cells were grown in serum-starved condition media for 24 h, treated with anti-IGF-IR blocking antibody at different concentrations (1, 2 and 5 μg/ml respectively) for overnight. Normal mouse IgG (5 μg/ml) was used as control. Real-time PCR was performed using primers of VEGF-C and 36B4 (internal control) with total RNA isolated from cells. In the figure, IgG stands for normal mouse IgG antibody and IGF-IR stands for anti-IGF-IR blocking antibody. The data here represent the mean of three different experiments. (**e**) VEGF-A mRNA level was unchanged in the presence of IGF-IR antibody. LNCaP cells were grown in serum-starved media for 24 h before treated with anti-IGF-IR-blocking antibody at different doses (1, 2 and 5 μg/ml respectively) for overnight. Normal mouse IgG (5 μg/ml) was used as control. Real-time PCR was performed using primers specific for VEGF-A and 36B4 (internal control) with total RNA isolated from LNCaP cells. The data here represent the mean of three different experiments

Burgering, 1999; Lin *et al*., 1997; Morris *et al*., 1996; Ogg *et al*., 1997). Therefore, inhibition of the IGF-IR signaling pathway should lead to the less phosphorylation of forkhead transcription factors, allowing them to enter the nucleus and promote the transcription of their target gene. Using the Genomatix software available on the internet (http://www.genomatix.de), we searched the known promoter region of VEGF-C for potential binding sites of transcriptional regulators and found several for the forkhead transcription factor, FOXO-1. To study whether FOXO-1 could indeed upregulate VEGF-C transcription, we transiently overexpressed FOXO-1 in LNCaP cells and used real-time PCR to monitor VEGF-C mRNA levels (Figure 4a). With the transient overexpression FOXO-1, we found a significant increase in VEGF-C mRNA levels. FOXO-1 protein overexpression was detected by Western blot with the anti-FLAG antibody (Figure 4b).

Various biological functions controlled by FOXO-1 include cell cycle arrest, detoxification of reactive oxygen species, repair of damaged DNA, and apoptosis (Kops and Burgering, 1999). Recent reports suggest that the presence of SIRT1 (mammalian homolog of yeast silencing information regulator 2 (Sir2) gene) enhances the expression of the FOXO target genes involved in stress resistance but diminishes the expression of proapoptotic genes like Fas ligand and BIM. Sir2 has been shown to play a role in increasing the lifespan of yeast and *Caenorhabditis elegans* and is conserved throughout evolution. It is a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase. Forkhead transcription factors have been shown to be a target of SIRT1. Deacetylation of FOXO transcription factors by SIRT1 is specifically required for the transcription of stress resistance genes, such as genes that control the repair of damaged DNA (GADD45) (Araki *et al*., 2004; Brunet *et al*., 2004; Cohen *et al*., 2004; Daitoku *et al*., 2004). Because the VEGF family is known to be involved in lymphangiogenesis, angiogenesis, and survival, we studied the involvement of SIRT1 in the FOXO-1-dependent transcription of VEGF-C. We found that BML-210, a specific SIRT1 inhibitor, abrogated FOXO-1 dependent VEGF-C transcription (Figure 4c). This result was confirmed when we used the dominant-negative form of SIRT1 to inhibit its activity (Figure 4d). SIRT-1DN protein overexpression was detected by Western blot with the anti-FLAG antibody (Figure 4e). Since androgen withdrawal leads to inhibition of PI3K-AKT pathway (Baron *et al*., 2004; Castoria *et al*., 2004; Kang *et al*., 2004) and therefore potentially activates FOXO-1, we tested whether dominant-negative form of SIRT-1 could abrogate the increase in VEGF-C expression in LNCaP due to androgen withdrawal. Our result (Figure 4f) clearly indicated the involvement of SIRT-1 in androgen-regulated VEGF-C transcription. These results are significant as they provide a mechanistic explanation for how FOXO-1, activated by SIRT1, can act to

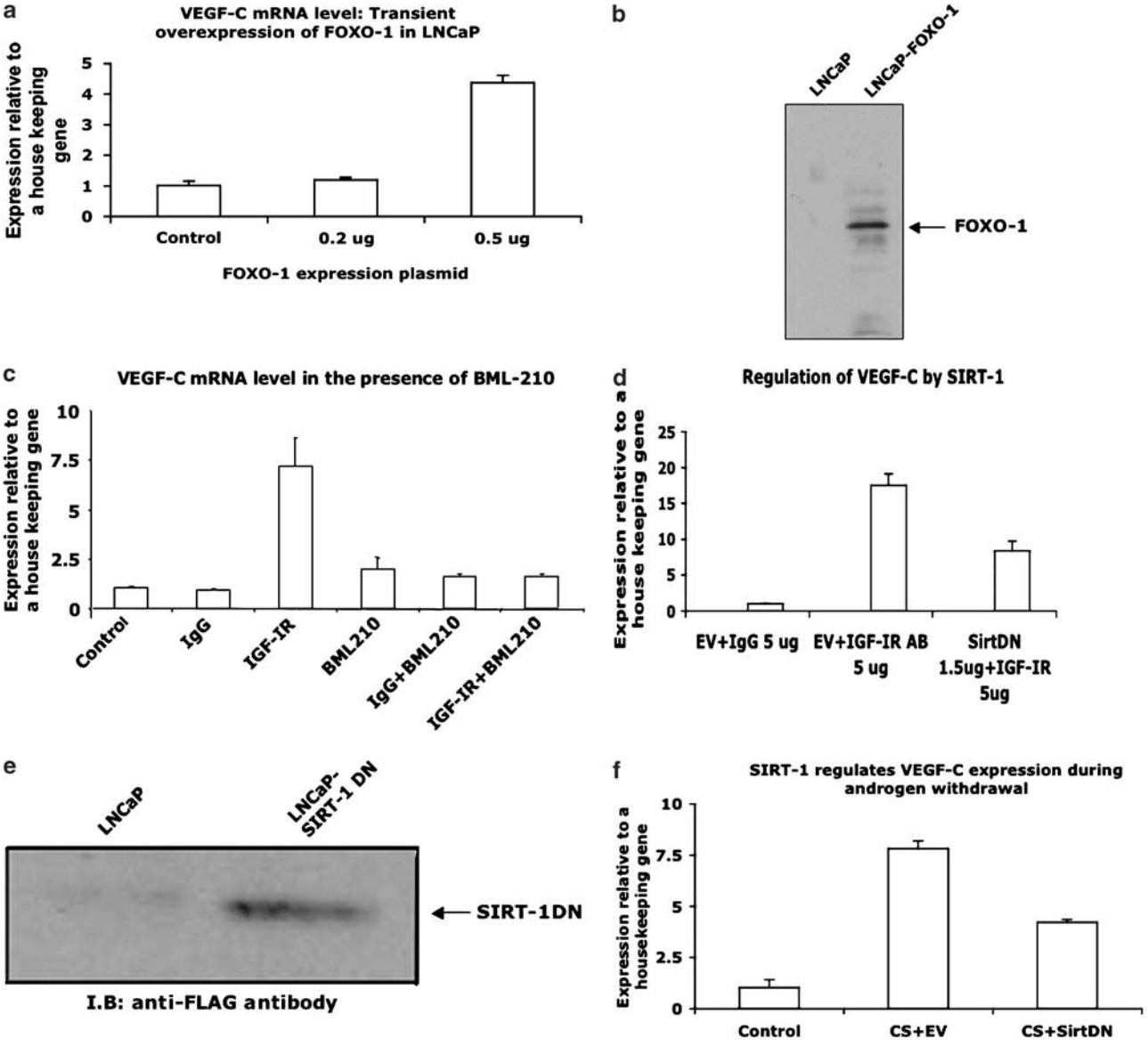

Figure 4 FOXO-1 with SIRT1 upregulates VEGF-C transcription. (**a**) LNCaP cells transiently transfected with pcDNA3–Flag–FOXO-l plasmid at 0.2 and 0.5 μg for 48 h, total RNA were subjected to real-time PCR using specific primers of VEGF-C and 36B4 (internal control), an increased mRNA level of VEGF-C was observed in LNCaP cells where FOXO-1 was overexpressed. The data here represent mean of three separate assays. (**b**) Western blot was performed using anti-flag antibody with the whole cell lysate extracted from the LNCaP cells transiently transfected with 0.5 μg of Flag-FOXO-1 plasmid. (**c**) FOXO-1-dependent VEGF-C transcription is abrogated by BML-210 (SIRT1 inhibitor). LNCaP cells were grown in 0.1% FBS for overnight. Anti-IGF-IR blocking antibody (5 μg/ml) either alone or along with BML-210 (10 μM) was then added for 8 h. Normal mouse IgG (5 μg/ml) was also used separately either alone or along with BML-210 (10 μM) for control experiment. Total RNA was isolated for each set and real time PCR was performed for VEGF-C and 36B4 (internal control). In the figure, IgG stands for normal mouse IgG antibody and IGF-IR stands for anti-IGF-IR blocking antibody. The data here represent mean of three separate assays. (**d**) Overexpression of dominant-negative form of SIRTl decrease the VEGF-C mRNA level. LNCaP cells transiently transfected with plasmid expressing dominant-negative form of SIRT1 (1.5 μg, pECE-SIRT-lDN)) in the presence or absence of anti-IGF-IR antibody (5 μg/ml). Total RNA was extracted from LNCaP cells and mRNA level of VEGF-C was determined by RT–PCR using primers specific for VEGF-C and 36B4 (internal control). In the figure, EV represents empty vector, pECE. IgG stands for normal mouse IgG antibody and IGF-IR stands for anti-IGF-IR blocking antibody. The data here represent mean of three separate assays. (**e**) Western blot was performed using anti-flag antibody with the whole cell lysate extracted from the LNCaP cells transiently transfected with 1.5 μg of Flag-SIRT-IDN plasmid. (**f**) Overexpression of dominant-negative form of SIRT1 decrease the VEGF-C mRNA level induced by androgen depletion. LNCaP cells transiently transfected with either plasmid expressing dominant-negative form of SIRT1 (1.5 μg) or empty vector (EV) and cultured in the presence of charcoal–dextran-treated serum-containing media for 72 h. For control experiment, LNCaP cells were cultured in the normal serum-containing media. Total RNA was extracted from LNCaP cells and mRNA level of VEGF-C was determined by RT–PCR using primers specific for VEGF-C and 36B4 (internal control). The data here represent mean of three separate assays

transcribe VEGF-C under androgen withdrawal condition and it also indicates that VEGF-C is another target gene for SIRT1.

VEGF-C can increase the expression of the transcription coactivator BAG-IL in LNCaP

VEGF family members are multifunctional growth factor proteins. The established function of VEGF-C is to potentiate the growth and sprouting of lymphatic endothelial cells or lymphangiogenesis by binding its receptor VEGFR-3. It is therefore important to determine the contribution of lymphangiogenesis to the survival and metastasis of prostate cancer cells during their transition to the androgen-refractory stage. In a recent report, Rafi *et al*. showed that expression of the antiapoptotic protein Bcl-2 was increased in the leukemia cells after treatment with VEGF-C (Dias *et al*., 2002) suggesting a function distinct from lymphangiogenesis. In the prostate cancer cell line LNCaP, the mRNA level of VEGFR3 was low and at least 10-fold lower than VEGFR2 as detected by real-time PCR (Figure 5a). The relative expression levels of these two receptors in the lymphatic endothelial cells (kind gift from Dr Mihaela Skobe) were also tested as a control experiment (Figure 5b). Figure 5a therefore indicated a potential VEGF-C–VEGFR-2-mediated autocrine pathways in LNCaP. A similar autocrine loop for VEGF-A–VEGFR2 in LNCaP cells were also reported by Steiner *et al*. (2004). Although VEGF-C is known to bind VEGFR-2, the significance of this binding is not known. Interestingly, we observed an increase in the expression of Bag-1 long isoform (Bag-IL) after treating serum-starved LNCaP cells with recombinant VEGF-C protein for 24 h (Figure 5c). Several reports suggested that the transcriptional coactivator, Bag-IL enhances the transactivation function of androgen receptor (Froesch *et al*., 1998; Shatkina *et al*., 2003). Therefore, an increase in Bag-IL protein level by VEGF-C may therefore provide an explanation of VEGF-C-mediated androgen receptor activation in the low concentration of androgen.

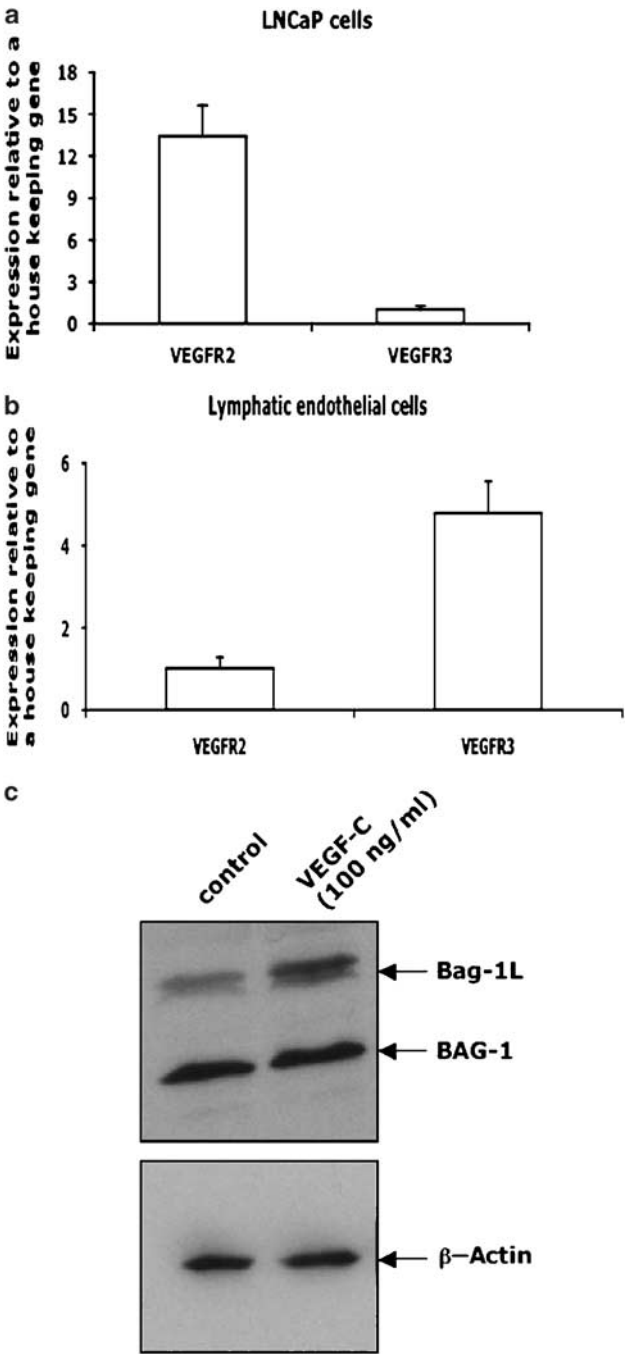

Figure 5 (**a** and **b**) VEGFR2 is predominant in LNCaP cells compare to VEGFR3. Real-time PCR was performed using specific primers for VEGFR2, and VEGFR3 with total RNA isolated from (**a**) LNCaP cells and (**b**) lymphatic endothelial cells (kindly provided by Dr Mihaela Skobe). The data represented here are the average of three independent results. (**c**) Recombinant VEGF-C protein increases the protein level of long isoform of Bag-1 (BaglL). Serum-starved LNCaP cells were incubated with recombinant VEGF-C protein (100 ng/ml) for 24 h. The proteins of the whole cell lysates were resolved by SDS–PAGE followed by Western blot using antibody specific for Bag-1. This antibody also recognizes Bag-IL (the long isoform) at 58 kDa. Membrane was then stripped for Western blot against β-actin (lower panel). The Western blot was repeated more than three times

Discussion

A detail molecular understanding of the gradual transition of prostate tumors from androgen dependence to androgen independence during androgen ablation therapy is necessary to design an effective therapy against this stage of the disease. Although some mechanisms have been postulated for androgen receptor activation, the involvement of angiogenic growth factors provides an alternative avenue of study. It is possible that angiogenic growth factors may act independently or in conjugation with the androgen receptor, to regulate the survival and metastasis of the androgen-refractory stage of prostate cancer. The possible crosstalk between the androgen receptor and angiogenic growth factors was first established by observing that androgens may modulate angiogenesis in animal and human prostate tumors, potentially by increasing vascular endothelial

growth factor-A (VEGF-A) gene transcription (Jain *et al*., 1998; Stewart *et al*., 2001). As a result, an initial and rapid vascular regression occurs after hormone withdrawal in prostate cancer. Surprisingly, however, progression of prostate cancer into an androgen-independent stage is also associated with increased angiogenesis. Thus far, the mechanism of this second wave of angiogenesis is unknown.

Interestingly, VEGF-C, another VEGF family member, is also expressed in human metastatic prostate cancer specimens. An increased number of vessels positive for VEGFR-3, the VEGF-C receptor, has been reported in the surrounding stromal tissue of VEGF-C positive prostatic carcinoma cells (Skobe *et al*., 2001; Zeng *et al*., 2004). Taken together, these reports suggest the importance of VEGF-C in prostate cancer metastasis. We have observed an increase in VEGF-C mRNA and protein in the more metastatic prostate cancer cell lines PC3 and DU145 as compared to LNCaP. Even the more metastatic sublines of LNCaP, LNCaP-LN3 and LNCaP-Pro5 expressed higher levels of VEGF-C compared to the parental line. Our focus in this study was to understand the mechanism of VEGF-C expression in prostate cancer, specifically in the context of androgen-deprivation conditions. We observed a time-dependent increase in VEGF-C mRNA levels when LNCaP cells were cultured in media containing charcoal–dextran-treated serum (reduced androgen containing serum). This increase in VEGF-C mRNA levels was inhibited after supplementing the media with the synthetic androgen R1881. This result therefore confirms the previous findings (Ruohola *et al*., 1999) that androgens have a negative regulatory effect on VEGF-C expression and suggests a potential functional significance for higher VEGF-C levels during the initial stage of androgen withdrawal. Interestingly, we observed a decrease in VEGF-A mRNA levels in LNCaP cells upon androgen depletion, confirming previous findings that androgen positively regulates VEGF-A mRNA transcription. These observations describe a potentially unique role for VEGF-C in androgen regulation.

Insight into a possible cellular mechanism for the elevation of VEGF-C levels under androgen-depleted conditions came with the observation that inhibition of the IGF-IR signaling pathway in LNCaP cells lead to the upregulation of VEGF-C mRNA. A recent report suggested that androgen stimulation of LNCaP cells cultured in media containing charcoal–dextran-treated serum leads to an increase in IGF-IR protein expression (Arnold *et al*., 2005). The activation of PI3K pathway by androgen was also reported previously (Baron *et al*., 2004; Castoria *et al*., 2004; Kang *et al*., 2004). These reports, together with our observation of higher VEGF-C mRNA levels after inhibiting IGF-IR signaling supports the conclusion that androgen deprivation leads to a decrease in IGF-IR expression that in turn increases the transcription of VEGF-C. This negative regulation of VEGF-C transcription by IGF-IR might be specific for prostate cancer. Recently, Tang *et al*. (2003) demonstrated a positive regulation of VEGF-C transcription by IGF-IR in Lewis lung carcinoma subline, M27. These differences in IGF-IR signaling for VEGF-C transcription therefore pointed out the heterogeneity of cellular signaling in different cancers and also in the different stages of a particular cancer. FOXO-1 is a member of the forkhead transcription factor family whose transcriptional activity is negatively regulated by IGF-IR (Morris *et al*., 1996; Borkhardt *et al*., 1997; Lin *et al*., 1997; Ogg *et al*., 1997; Kops and Burgering, 1999) and is therefore a potential downstream target of IGF-IR in VEGF-C transcription. Interestingly, we have found several potential binding sites for FOXO-1 in the VEGF-C promoter region using Genomatix software. To show that FOXO-1 is the probable transcription factor for VEGF-C, we have overexpressed FOXO-1 in LNCaP cells. A significant increase in VEGF-C mRNA was observed in LNCaP cells overexpressing FOXO-1. The target genes for FOXO-1 include both the proapoptotic genes as well as the genes involved in stress resistance. Recently, a protein called SIRT1 has been shown to regulate FOXO-1 activity by enhancing the transcription of stress resistance genes but diminished the expression of proapoptotic genes such as Fas ligand and BIM. SIRT1 specifically deacetylates FOXO-1 in order to regulate its activity (Araki *et al*., 2004; Brunet *et al*., 2004; Cohen *et al*., 2004; Daitoku *et al*., 2004). The finding that VEGF-C transcription driven by FOXO-1 requires SIRT1 suggests that VEGF-C is another survival related target gene of FOXO-1. In this study, we have therefore suggested a possible mechanism of VEGF-C transcription resulting from androgen ablation in the prostate cancer cell line, LNCaP. Androgen ablation decreases IGF-IR expression and therefore disrupts its function. The resulting effect is activation of FOXO-1, which with SIRT1, increases the transcriptional activity of VEGF-C.

VEGF-C can potentiate different functions in prostate cancer, many of which may be responsible for its growth and survival in the androgen-withdrawal stage. Particularly, increased synthesis of VEGF-C may be linked to its early and mid-phase transition to the highly metastatic, and androgen-refractory stage. The main function of VEGF-C is its involvement in lymphangiogenesis where the growth and sprouting of lymphatic endothelium occurs from a pre-existing lymphatic vessel (Skobe *et al*., 2001). Increased lymphatic vessel formation surrounding a tumor may provide potential routes for tumor cells to metastasize to distant organs. As androgen withdrawal triggers tumor growth arrest and tumor apoptosis in the primary site, an increase in the metastatic potential therefore helps the tumor cells to migrate to a different location and survive. Interestingly, there are reports that suggest a significant correlation between the expression of VEGF-C and lymph node dissemination in human prostate carcinoma (Tsurusaki *et al*., 1999). Moreover, the number of vessels positive for VEGFR-3, the receptor of VEGF-C, was increased in the surrounding stromal tissue of VEGF-C-positive prostatic carcinoma cells (Tsurusaki *et al*., 1999). Therefore, it will be interesting to study the involvement of lymphangiogenesis in prostate cancer metastasis during its transition to the androgen-refractory stage.

As VEGF family members are usually multifunctional in nature, we were also interested in identifying VEGF-C functions distinct from lymphangiogenesis that may be responsible for prostate cancer progression. We tested for the presence of VEGFR2 and VEGFR3, two receptors for VEGF-C in LNCaP cell lines with real-time PCR. We detected a very low level of VEGFR-3 in LNCaP cells, but VEGFR-2 was in the detectable level and $\sim$10-fold higher than VEGFR-3 under our experimental conditions (Figure 5a). This result suggests a possible VEGF-C autocrine loop in prostate cancer cells and possibly some VEGFR-2 specific functions in LNCaP. The concept of autocrine loop for VEGF family members in prostate cancer cells were further supported by the recent report of Steiner *et al*. (2004). The authors reported VEGF-A–VEGFR2-mediated autocrine loop in LNCaP cells after prolonged IL6 treatment. Along these lines, LNCaP cells also provide a unique opportunity to study the specific functions of VEGF-C and VEGFR-2 that are currently unclear.

The finding that BAG-IL (long isoform of Bag-1) was increased in serum-starved LNCaP after VEGF-C treatment is also significant. Bag-IL is a member of the Bcl-2 family and plays a role in cell survival during stress conditions. Bag-l(Bcl-2-associated athanogene 1) is a family of proteins that associates with the molecular chaperone Hsp70 and serves as a nucleotide exchange factor for this protein. There are four mammalian isoforms of Bag-1; Bag-lL, Bag-IM, Bag-lS, and p29, generated by alternative translation initiation sites on the same mRNA. The human Bag-IL protein migrates as a 57–58 kDa protein in SDS–PAGE experiments. The Bag-1 proteins have multiple biological functions ranging from inhibition of apoptosis to modulation of the action of steroid receptors. Bag-IL has been reported to enhance the transactivation function of the androgen receptor with the help of Hsp70. Furthermore, Bag-IL is expressed in prostate cancer specimens (Froesch *et al*., 1998; Sadar *et al*., 1999; Shatkina *et al*., 2003). Therefore, a VEGF-C-induced increase in Bag-IL may enhance transactivation of androgen receptor even in low concentrations of androgen.

In conclusion, we have observed an increase in the VEGF-C level in prostate cancer cells due to the withdrawal of androgens. We have delineated a possible mechanism for VEGF-C synthesis in LNCaP cells involving the androgen–IGF-IR–FOXO-1 pathway. Finally, we also determined a potential function mediated by VEGF-C that may be important for the growth and survival of prostate cancer from the early phase of its transition to the androgen-refractory stage.

Materials and methods

Cell culture and reagents

Human prostate cancer cell line, LNCaP (ATCC # CRL-1740), DU-145 (ATCC # HTB-81), and PC-3 (ATCC #CRL-1435), were maintained in RPMI medium and Ham's F-12 medium separately with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA). LNCap-LN3 and LNCap-pro5 were kind gifts from Dr Curtis A Pettaway of MD Anderson. FOXO-1 expression vector was obtained from Dr Haoji Huang of Mayo Clinic. Human SIRT1 dominant-negative (DN) plasmid (pECE-SIRTlDN) was also a kind gift from Dr Michael E Greenberg, Children's Hospital and Harvard Medical School, Boston. *NLP-005* Methyltrienolone (R1881) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BML-210 was purchased from BIOMOL International L.P. (Plymouth Meeting, PA, USA).

Transfection and whole cell extract preparation

LNCaP cells (5×10^5 cells) were seeded in 60 mm dish 1 day before transfection. Transfection was carried out with Effectene Transfection Kit (Qiagen, Valencia, CA, USA). Briefly, 0.5 μg of the FOXO-1 expression plasmid was resuspended in EC buffer (75 μl) and 4 μl of Enhancer was added and incubated at room temperature for 5 min. Effectene (10 μl) was then added and the whole mixture was incubated for another 10 min. RPMI with 10% FBS (350 μl) was added to the DNA mixtures. At 48 h after transfection, cells were washed three times with PBS, whole cell extracts was prepared in accordance to the following protocol.

Preparation of whole cell extracts

LNCaP, DU145, PC-3, LNCap-LN3 and LNCap-Pro5 cells were washed twice with 10 ml of cold PBS, lysed with ice-cold RIPA lysis buffer (50 mM Tris (pH 7.5), 1% Nonidet p-40 (NP-40), 150 mM NaCl, 1 mM Na_3VO_4, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 mg/ml), 0.5% aprotinin, 2 mM pepstatin A), incubated on ice for 30 min, and centrifuged at 14 000 r.p.m. at 4°C for 10 min.

Western blot analysis

The whole cell extracts were separated by SDS–PAGE; immunodetection antibodies against VEGF-C (R&D System, Inc., Minneapolis, MN, USA), Flag (Sigma), IGF-IRα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Bag-1 (Zymed Laboratories Inc., South San Francisco, CA, USA) were used, followed by the secondary antibody incubation, and detected by ESL enhancer reagent from Amersham Biosciences Corp (Piscataway, NJ, USA).

ELISA

Cell culture supernatant was collected after growing the prostate cancer cells at serum-starved media for 24 h. VEGF-C was measured in the cell culture supernatant using a commercial ELISA kit (Zymed laboratories Inc.) according to the manufacturer's protocol. Briefly, after incubation 100 μl of sample media or different dilutions of standard in the plate at 37°C for 1 h, the wells were washed three times with washing buffer, 100 μl of labeled antibody solution was added to each well for a 30 min incubation at 4°C. For color development, TMB buffer (100 μl) was added into the wells and was incubated for 30 min at room temperature in dark, the reaction was stopped by adding 100 μl of Stop solution. Color intensity was measured by a plate reader at 450 nm. Data represent the average of three different assays.

Treatment of cells with antibody, androgen and specific SIRT inhibitor

Cells were pretreated with IGF-IRα antibody (1H7) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (Keller *et al*., 1993; De Meyts *et al*., 1995; Datta *et al*., 2000; Schlessinger,

2000), R1881, BML-210 (specific SIRT inhibitor) and recombinant VEGF-C protein at 37°C for different time points. Cells were serum starved before IGF-IRa antibody, BML-210 and recombinant VEGF-C protein treatment. For R1881, cells were cultured in charcoal–dextran-treated serum for 48 h. After washing twice with ice-cold PBS, cells were lysed using lysis buffer from the RNeasy Mini kit (Qiagen). Total RNA was extracted according to the RNeasy mini kit protocol.

RNA preparation and real-time PCR

After washing twice with ice-cold PBS, different cell lines LNCaP and other cells were lysed using lysis buffer from the RNeasy Mini kit (Qiagen). Total RNA was extracted according to the RNeasy mini kit protocol. We used the Taqman real-time PCR method. The sequence for forward, reverse and Taqman middle primers for human VEGF-C, VEGF-A, VEGFR2, VEGFR3 and for human 36B4 (housekeeping gene) were taken from the PubMed gene bank and synthesized (Integrated DNA Technology). 36B4 is a ribosomal RNA and ubiquitously expressed in all the cells. Owing to its housekeeping function, it is used as a control experiment for real-time PCR, Northern blot etc. VEGF-C forward: 5′- AGT GTC AGG CAG CGA ACA AGA -3′. VEGF-C reverse: 5′-CCT CCT GAG CCA GGC ATC TG -3′. VEGF-C middle primer: 5′- TGC CCC ACC AAT TAC ATG TGG AAT AAT CA -3′. VEGF-A forward: 5′-TAC CTC CAC CAT GCC AAG TG-3′. VEGF-A reverse: 5′-GAT GAT TCT GCC CTC CTC CTT-3′. VEGF-A middle primer: 5′-TCC CAG GCT GCA CCC ATG GC -3′. VEGFR2 forward: 5′-CAC CAC TCA AAC GCT GAC ATG TA-3′. VEGFR2 reverse: 5′-CCA ACT GCC AAT ACC AGT GGA-3′. VEGFR2 middle primer: 5′-TGC CAT TCC TCC CCC GCA TC-3′. VEGFR3 forward: 5′-CAA GGC CAA CAA CGG CAT-3′. VEGFR3 reverse: 5′-TCG ACG CTG ATG AAG GGG ATT-3′. VEGFR3 middle primer: 5′-CAC AAT GAC CTC GGT GCT CTC CCG-3′. 36B4 forward: 5′-ATG CAG CAG ATC CGC ATG T-3′. 36B4 reverse: 5′-TCA TGG TGT TCT TGC CCA TCA-3′. 36B4 middle primer: 5′-CAC CAC AGC CTT CCC GCG AA-3′. Each real-time PCR reaction was conducted using 0.5 mg total RNA, 25 μl RT–PCR Master Mix (Applied Biosystems), 1.25 μl RNase inhibitor (Applied Biosystems), 50 nM forward primer, 50 nM reverse primer, and 100 nM middle primer. For reverse transcription, a 30-min period at 48°C was run before inactivating the reverse transcriptase at 95°C for 10 min. A total of 40 cycles at 95°C for 15 s and 60°C for 1 min was performed with an ABI Prism 7700 Sequence Detector (Applied Biosystems). All experiments were carried out three times and from each of the three, triplicate readings were taken and the average was calculated.

Relative RNA amount was calculated as follows: The cycle number (CT) at which PCR amplification took place in a particular threshold value in the exponential phase for each reaction was determined by the sequence detector. Real-time PCR for the housekeeping gene, 36B4 was performed for each test sample along with VEGF-C or VEGF-A. To normalize the value of VEGF-C or VEGF-A for each reaction condition, the value of 36B4 at that condition was deducted and the resulting value was designated as Δ. Therefore, $\Delta = $ CT (VEGF-C or A sample) − CT(36B4 sample). To calculate the relative expression of the treated sample compare to the control sample, first $\Delta\Delta$ was determined by deducting the Δ value of the control sample from the treated sample. Therefore, $\Delta\Delta = \Delta$ (transfected or treated sample) − Δ(empty vector or untreated sample). Finally, relative RNA amount in comparison to the control was calculated by using the formula, $2^{-\Delta\Delta}$. Average and standard deviations from three experiments were calculated.

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